

Optimizing a Municipal Wastewater-based *Chlorella vulgaris* Photobioreactor for Sequestering Atmospheric CO₂

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Abstract.—Microalgae photobioreactors are among the most effective systems for capturing gaseous CO₂, the main contributor to global warming. Their capacity to generate massive amounts of biomass has been exploited serendipitously to sequester CO₂ and explicitly to remove nutrients from municipal wastewater. Unfortunately, research in this area has not included merging these dual capacities to address global warming. Instead, most are focused on thermolytic conversion of biomass into energy which in end returns CO₂ to the atmosphere. In this study, we investigated the potential of combining the two microalgal capacities (that of deriving nutrients from municipal wastewater and metabolic carbon from atmospheric or industrial CO₂ supplies), into an integrated means of reducing nutrients in ocean-bound wastewater and CO₂ in the atmosphere simultaneously. The test species used in this study was *Chlorella vulgaris* (*C. vulgaris*); the turbidity of *C. vulgaris* was used as a measure of yield in biomass. Our results show (i) that an open photobioreactor, and not a closed one, is the most productive, especially when augmented with industrial CO₂ (hence making a strong case for scrubbing CO₂ gas from industrial sources), (ii) that a mechanically agitated *C. vulgaris* culture is more productive than a static one, (iii) that without mechanical agitation, 32 ± 3 days of incubation are needed to reach the maximum yield of an open photobioreactor, (iv) that the optimal proportion of wastewater (%WW) required to support *C. vulgaris* growth is $80 \pm 3\%$; at least 33% WW is required to observe growth above background, and (v) that without intervention, the upper pH limit of a WW-based *C. vulgaris* culture is 8.69 ± 0.09 . Two mutually independent models are proposed to aide in scaling up an open WW-based *C. vulgaris* photobioreactor.

Global warming and eutrophication are two of the most pressing challenges confronting environmental scientists in recent times (Committee on Science for EPA's Future 2012). Global warming, the steady heating of the earth surface and of the atmosphere, is partly a consequence of atmospheric emission of greenhouse gases such as carbon dioxide (CO₂), methane (CH₄), nitrous oxide (N₂O), and some fluorinated compounds (Rey et al. 2018). These greenhouse gases do not only absorb and retain infra-red energy (Rey et al. 2018), they prevent the release of heat into the outer space from the earth surface (Akitt 2018). Of these gases, CO₂ is implicated the most in global warming (reviewed by Anderson et al. 2016). In 2015 alone, 82% of all greenhouse gases emitted was CO₂.¹ In modern times,

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¹ Olivier J.G.J., G. Janssens-Maenhout, M. Muntean and J.A.H.W. Peters. 2016. Trends in global CO₂ emissions. The Hague: PBL Netherlands Environmental Assessment Agency. Available

the main source of CO₂ is the combustion of fossil fuel to meet domestic and industrial energy needs (Fischer et al. 2018; Carotenuto et al. 2018). Efforts to curb global warming henceforth will, therefore, have to include controlling this anthropogenic CO₂ emission alongside reducing CO₂ levels already present in the atmosphere.

Eutrophication, the excessive plant growth in large water bodies, is due to increased availability of sunlight, carbon dioxide, and nutrients (Greeson 1969; Schindler 2006). This excessive plant growth in turn creates areas in aquatic environment where the amount of dissolved oxygen is not enough to support marine life. In current times, the discharge of large amounts of nitrogen and phosphorus into the aquatic ecosystems has increased the rate of eutrophication and the extent to which eutrophication is affecting inland waterways and coastal waters (Carpenter et al. 1998; Chislock et al. 2013). The affected areas (also referred to as dead zones) have been shown to occur frequently near where agricultural runoff and wastewater treatment effluent are released into large water bodies.² Efforts to remediate eutrophication resulting from these anthropogenic activities may have to include polishing agricultural runoffs and municipal wastewater before discharge.

Over the last few years, microalgae have emerged as promising vehicles for converting CO₂ into large quantity of biomass. Research in this area though is primarily focused on optimizing the biomass production for the biofuel industry with little considerations being given to the consequential release of the CO₂ back into the atmosphere (Xu et al. 2006; Sayre 2010; Mondal et al. 2017). In our opinion, the biomass so produced could be stored to control the amount of gaseous CO₂ in circulation. Storage could mean delaying or preventing the release of CO₂ into the atmosphere. Others have proposed chemical means (Obersteiner et al. 2001; Lackner et al. 2012; Wurzbacher et al. 2012) and the ocean (Shaffer 2010) as solutions towards reducing the amount of CO₂ in circulation. However, chemical means would require careful attention to the disposal of the chemical wastes so generated in the process, and both legal and environmental hurdles will have to be overcome before anthropogenic CO₂ is stored in the ocean.

In this study, we propose a natural way of sequestering CO₂ in biomass. We investigate whether a stable culture of microalgae with the following characteristics can be maintained in a mixotrophic photobioreactor: (i) one that derives metabolic carbon from CO₂ (Sayre 2010; Mondal et al. 2017; Raven 2017) and (ii) one that would also utilize secondary-treated municipal wastewater as the only source of nutrients (Lau et al 1995; Wang et al. 2010; Craggs et al. 2011; Shi et al. 2016). We believe that a successful integration of these two microalgal natural capacities can play a role in scrubbing CO₂ gas not only from the atmosphere but from industrial sources as well while removing nutrients from ocean-bound municipal wastewater (WW) at the same time. This, metaphorically, is killing two birds with the same stone. The removal of nutrients in particular is of importance to watersheds and municipal wastewater treatment plants that discharge freshwater and treated wastewater effluents, respectively into large water bodies. Elevated level of anthropogenic nutrients in the ocean for example is known to promote toxic algal blooms (Anderson et al. 2002).

The key factors investigated in this study are those known to affect the productivity of microalgae photobioreactor. These factors include microalgae species to culture, the

from: http://edgar.jrc.ec.europa.eu/news_docs/jrc-2016-trends-in-global-co2-emissions-2016-report-103425.pdf. Accessed 05 July, 2018.

² Paine V. 2012. What Causes Ocean “Dead Zones”? Sci. Am., Available from: <https://www.scientificamerican.com/article/ocean-dead-zones/>. Accessed 15 January, 2019.

Table 1. Composition of secondary-treated wastewater used in this study. [†]

Test	Results
pH	7.3
Carbonaceous Biochemical Oxygen Demand	5 mg/L
Biological Oxygen Demand	12 mg/L
Settleable Solids	<0.1 mL/L
Turbidity	5.1 NTU
Mercury, Hg	0.00239 µg/L
Silver, Ag ^a	0.061 µg/L
Boron, B	0.541 mg/L
Antimony, Sb	2.75 µg/L
Beryllium, Be ^a	0.03 µg/L
Cadmium, Cd ^a	0.05 µg/L
Copper, Cu	13.4 µg/L
Nickel, Ni	6.52 µg/L
Thallium, Th ^a	0.02 µg/L
Zinc, Zn	14.8 µg/L
Arsenic, As	2.30 µg/L
Selenium, Se ^a	0.52 µg/L
Total Suspended Solids	9.7 mg/L
Total Dissolved Solids	768 mg/L
Total Organic Carbon	19.1 mg/L
Total Kjeldahl Nitrogen	47.8 mg/L
Organic Nitrogen	<1 mg/L
Total Phosphate	2.82 mg/L
Ammonia as Nitrogen	47.0 mg/L
Nitrate as Nitrogen	<0.1 mg/L
Nitrite as Nitrogen ^a	0.32 mg/L
Chloride	303 mg/L

[†] Available data at: <http://ciwqs.waterboards.ca.gov/ciwqs/readOnly/PublicReportEsmrAtGlanceServlet?reportID=2&isDrilldown=true&documentID=1893752>.
^a Values lie between detection limit and regulatory reporting limit.

method of delivering CO₂ to the culture, the nutritional needs of microalgae, the pH changes occurring during incubation in the microalgae culture, and photobioreactor configuration (i.e., an open-air system versus a closed one; most researchers use the latter without provision for releasing metabolic O₂ known to be toxic to microalgae). Earlier studies in our lab (unpublished) suggests that *C. vulgaris* can flourish under the mixotrophic cultivation setup adopted for this study. The potential of using *C. vulgaris* similarly has been demonstrated recently by Shi et al. (2016), and the kinetics of *C. vulgaris* growth in freshwater has also published by Adamczyk et al. (2016).

Materials and Methods

All containers used in this study were thoroughly washed with soapy water, rinsed with distilled water (DH₂O) and disinfected with 3% aqueous solution of hydrogen peroxide (PL Development, Clinton, SC, USA). Secondary-treated wastewater was acquired from the Hyperion Water Reclamation Plant (City of Los Angeles, CA, USA); its parameters, listed in Table 1, were determined as described elsewhere (Otim et al. 2018).

Chlorella vulgaris cultures (10 mL in Alga-Gro Freshwater growth medium) were purchased from Carolina Biological Supply (Burlington, NC, USA) and cultured using a

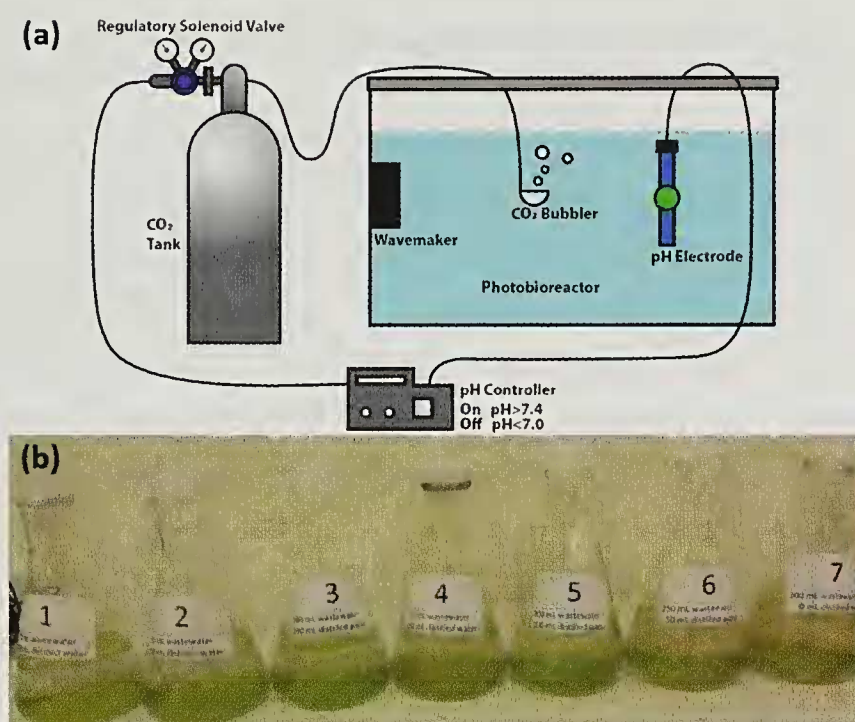


Fig. 1. (a) The setup used to maintain a viable stock of *Chlorella vulgaris* in distilled water. (b) Cultivating *Chlorella vulgaris* in secondary-treated wastewater (WW) collected from the City of Los Angeles Hyperion's 5-Mile Outfall system. The wastewater was serially mixed (in mL of WW) with distilled water to a final volume of 310 mL in each 500-mL flask (or 500-mL beaker, not shown) as follows: *Flask 1*: 0 (0% WW), *Flask 2*: 50 (16% WW), *Flask 3*: 100 (33% WW), *Flask 4*: 150 (50% WW), *Flask 5*: 200 (67% WW), *Flask 6*: 250 (83% WW), and *Flask 7*: 300 (100% WW). The 310 mL total volume includes 10 mL of laboratory stock of *Chlorella vulgaris* (see Materials and Methods section). Glass wool and perforated saran wraps were used to cover the open mouths of Erlenmeyer flasks and beakers, respectively.

modification of James (2012) protocol as follows. The *C. vulgaris* cultures were first stored as received at room temperature under an alternating cycle of artificial white light (16 h) and darkness (8 h) for seven days to allow acclimatization. Then before use, the cultures were diluted to 1 L with fresh Alga-Gro Freshwater growth medium and incubated for seven more days under alternating cycle of artificial white light as described above. To maintain a laboratory stock, *C. vulgaris* was cultured in an open photobioreactor (30-L DH₂O) fitted with the capacity to boost the level of CO₂ in microalgae culture automatically via a gas dispersion frit to bring down pH to 7.0 when necessary (Fig. 1a); the upper limit of pH was set to 7.4. Nutrients (20 mL of Guillard's (F/2) Marine Water Enrichment Solution; Carolina Biological Supply, Burlington, NC; Guillard and Ryther 1962; Guillard 1975) were supplied to the *C. vulgaris* laboratory stock every seven days.

To determine the ratio of wastewater-to-distilled water (WW/DH₂O) suitable for optimal growth, two separate trials of seven 310-mL photobioreactors were set up in 500-mL beakers (not shown) and in Erlenmeyer flasks shown in Fig. 1b (labeled 1 to 7) to assure reproducibility. This dual setup also served to gauge in a rudimentary way the effect of exposing different surface areas on photobioreactor performance. The WW/DH₂O ratios were formulated as follows (v/v): 0/300, 50/250, 100/200, 150/150, 200/100, 250/50, 300/0. Into each photobioreactor was added 10 mL of laboratory stock *C. vulgaris* (which brought the total volume to 310 mL) and, without stirring, the microalgae were allowed to grow as lawns. The endpoint for these experiments came when the green microalgae began to turn brown (beakers: 34 days; flasks: 29 days). Microalgal yield was measured as changes in turbidity (NTU) using a NUL-231 Turbidity Logger Sensor coupled to a USB-200 USB Module (Rochester, NY, USA). No external supplies of CO₂ or nutrients

were administered in these two sets of cultures beyond atmospherically or wastewater supplied (Table 1), respectively.

To measure and/or control the pH of microalgae cultures, an integrated pH monitoring system (Doctors Foster and Smith, Rhinelander, WI, USA) was used. The components of the system were (i) a Pintpoint pH electrode (measurement range: 1.00–14.00; resolution: 00.00 pH Unit; America Marine Inc., Ridgefield, CT, USA), (ii) a pair of CO₂ delivery regulators (Azoo, Taipei, Taiwan) controlled by a single solenoid valve (Jin Ben Sun Co., Ltd., Taoyuan City, Taiwan), and (iii) a Pinpoint pH Controller (America Marine Inc., Ridgefield, CT, USA) for ultimately powering, monitoring and controlling all the components of the system. Before using the automated pH monitoring system for acquiring experimental values, a two-point pH electrode calibration was carried out using a pH 7.00 certified reference buffer solution (to set the lower detection limits) and a pH 10.00 certified reference buffer solution (to set the slope). Both certified buffer solutions were supplied by Fisher Chemicals (Fair Lawn, NJ, USA). To monitor and regulate the pH of 30-L photobioreactors, the integrated system was configured as shown in Fig. 1a. The pH electrode was cleaned and recalibrated when necessary (usually once a week). To monitor the pH of the seven 310-mL photobioreactor flasks (Fig. 1b) and beakers (not shown), the pH electrode was used to gently stir the microalgae cultures for about 30s before taking measurements. The electrode was rinsed with distilled water between measurements. By design, the 310-mL photobioreactors required no additional CO₂ gas supplies.

To determine the empirical differences between an open microalgae photobioreactor system and a closed one by turbidity measurements, four separate 30-L tap water-based *C. vulgaris* photobioreactors similar to that shown in Fig. 1a were set up as follows. The first two were open to the atmosphere with one receiving additional supply of industrial CO₂ (Open, CO₂) and the other receiving no additional CO₂ supply beyond atmospherically supplied (Open, No CO₂). The third and the fourth photobioreactors were closed systems but similarly configured (i.e., Closed, CO₂ and Closed, No CO₂). Tap water (pH 7.27 ± 0.09 , $n = 4$) was used here because it is the background matrix of the wastewater used in this study. To each photobioreactor was added a 1 L dilution of the stock *C. vulgaris* as described earlier in this section, and 20 mL of Guillard's (F/2) Marine Water Enrichment Solution (Carolina Biological Supply, Burlington, NC, USA) every seven days. Each photobioreactor was equipped with a circulation pump (wavemaker, Fig. 1a) to keep microalgae in suspension. These four pumps, discovered to increase the temperature of the microalgae cultures, were turned on and off automatically every 30 min to keep the temperature of the photobioreactors to within $23.1 \pm 0.2^\circ\text{C}$. The light cycles and the pH of cultures were monitored and controlled as described earlier in this section. Turbidity was measured every seven days, and on the 10th day, over 21 days.

Non-linear modeling of turbidity and pH variation with time, and principal component analysis (PCA) were performed using the PAST software platform (PAleontological STatistics; Hammer et al. 2001). PCA, an unsupervised linear dimensionality reduction algorithm (reviewed by Jolliffe and Cadima 2016; see also Gewers et al. 2018) was used to find a more meaningful coordinate system to work with since our variables have different units (i.e., %WW, incubation days and pH). It was used similarly to study growth and biochemical composition of *C. vulgaris* in different growth media by Chia et al. (2013). Standardizing data for PCA was accomplished by subtracting the mean value from individual measurement and dividing the result by standard deviation.

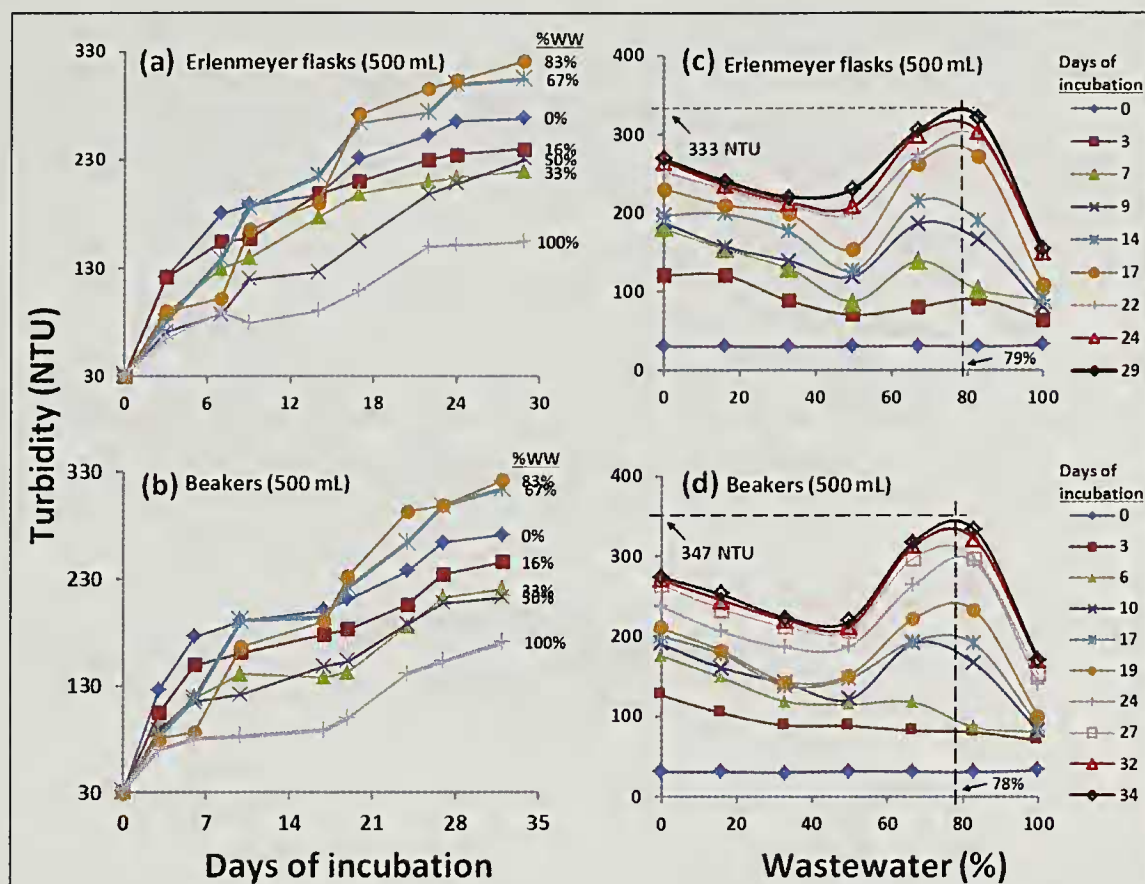


Fig. 2. The profiles of *Chlorella vulgaris* growth as measured by the turbidity of the seven wastewater/distilled water mixtures described in Fig. 1b. For cross authentication, duplicate test cultures were started on different dates with two different sets of container geometries to run for 29 days in 500-mL Erlenmeyer flasks (a) and 34 days in 500 m-L beakers (b). The end of incubation was determined by the browning of the microalgae in the cultures. Experimentally determined maximum turbidities are shown by the intersections of the horizontal dash lines and the ordinate in (c) and (d). Also shown at the intersection of each vertical dash line with the abscissa is the optimal %WW. The experimental coordinate for the maximum turbidity in the Erlenmeyer flasks (c) is (79%, 333 NTU) and in the beakers (d) is (78%, 347 NTU). Modelling (not shown) suggests that the coordinate for maximum turbidity should actually be (83%, 773 NTU).

Results

To determine the optimal WW/DH₂O ratio and incubation time required to maximize biomass yield, *C. vulgaris* was cultured in a pair of serially diluted wastewater photobioreactors for 29 and 34 days. Results show that yield, as measured by the turbidity of the cultures, is reproducibly related to time and exposed surface area (compare Erlenmeyer flasks results displayed in Fig. 2a with beakers results in Fig. 2b). The highest turbidity values were measured after 15 days of incubation in the 83% WW culture; the lowest being recorded in the 100% WW irrespective of the length of incubation time.

To acquire potentially useful information for scaling up a wastewater-based photobioreactor, a non-traditional interpretation of our data is provided in Figs. 2c-d. Four conclusions can be made from these two plots: (i) that 100% DH₂O or 100% WW is not the best medium for culturing *C. vulgaris* even though 100% DH₂O is more accommodating than 100% WW, (ii) that yield falls with increasing %WW during the first six days of incubation, (iii) that 80 ± 3% WW culture is the optimal range for realizing the most biomass in wastewater provided incubation is allowed to continue for at least 29 days (but not more than 34 days because the green microalgae turns brown thereafter under this condition), and (iv) that beyond 78-83% WW, wastewater severely limits *C. vulgaris* growth.

To understand the ability of *C. vulgaris* to absorb atmospheric CO₂, we monitored changes in pH in each of the WW/DH₂O cultures over time. The rationale was that pH is directly related to the availability of dissolved CO₂ in the cultures (Chi et al. 2011) which

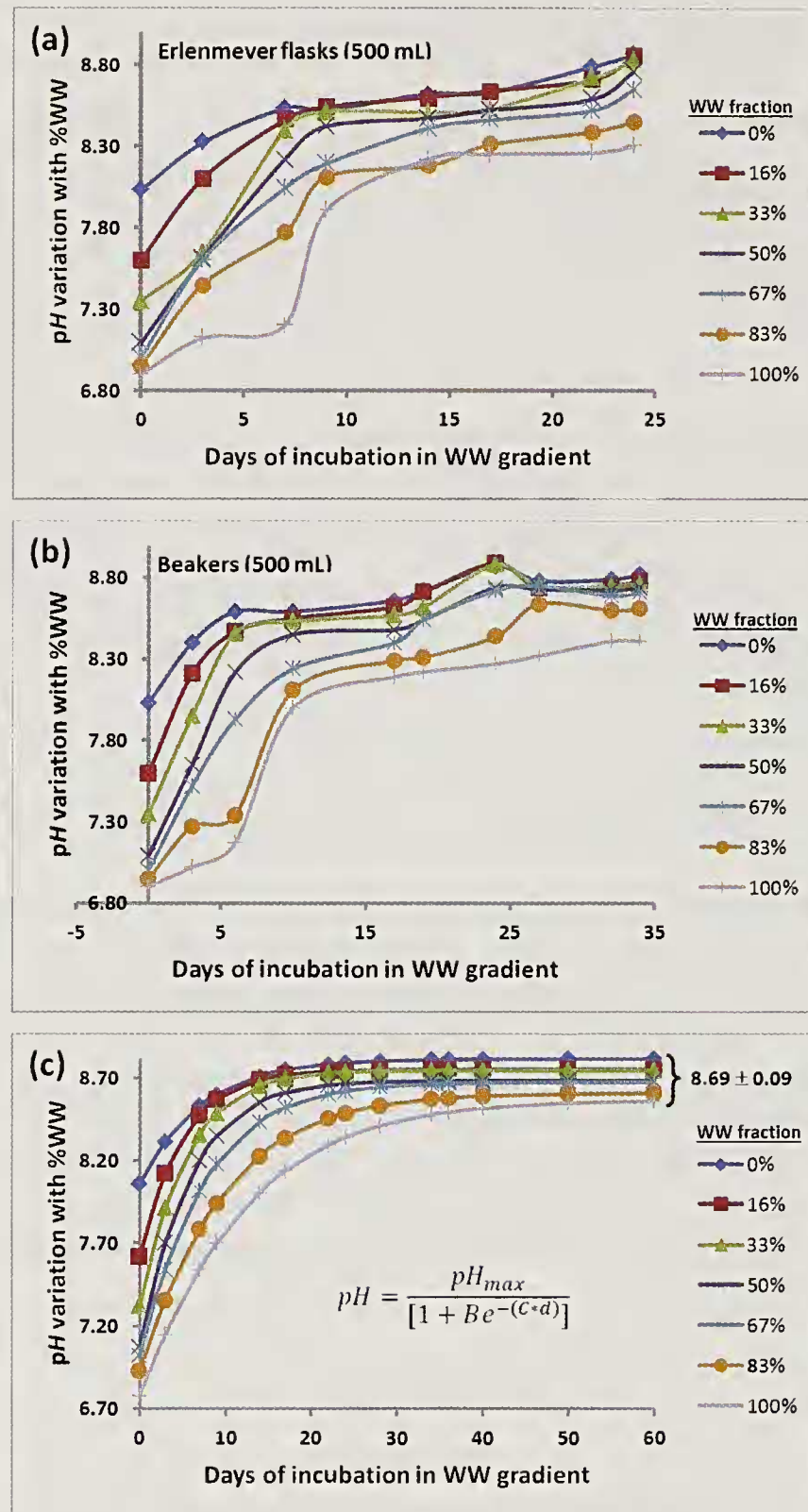


Fig. 3. The variation of pH with time in the WW-based photobioreactors at different %WW: (a) 500-mL Erlenmeyer flasks; (b) 500-mL beakers. The duplicate experiments (a) and (b) were started at different times for cross authentication. (c) Modeling the pH variation in (a) and (b) to determine the theoretical pH maximum (i.e., $pH_{max} = 8.69 \pm 0.09$). Note (i) that the $pH_{max} = 8.69 \pm 0.09$ is also the mean of pH_{max} values in Table 3, and (ii) that the inflection points in (c) are near constant in growth media containing 0% to 33% WW, but vary widely between 50% and 100% WW.

can in turn be directly linked to the capacity to capture atmospheric CO_2 . Duplicate results from this pH study are presented in Fig 3a where eight pH measurements were taken on different days over a period of 29 days and in Fig. 3b where 10 measurements were taken similarly over a period of 34 days. In both duplicates, the lowest pH values were measured in 100% WW culture, and the highest in the 0% WW culture (DH_2O , $pH = 7.99 \pm 0.06$, $n = 2$). It can also be seen in both cases that an upper pH ceiling is reached in each %WW culture after 10 days of incubation. The average pH value at these ceilings is 8.7 ± 0.1 ($n = 14$). This average value reveals the enormous potential of a wastewater-based photobioreactor for absorbing CO_2 .

Data from the open WW photobioreactor system (Fig. 1) show that the relationship between turbidity (τ) and the length of incubation in days (d) in any of our

Table 2. Values of constants τ_{max} and D in the Michaelis-Menten growth equation $Turbidity = [\tau_{max} * d] / [D + d]$ derived from regression analysis of turbidity data at each %WW (Fig. 2a-b). They are reported as mean±standard deviation.

	0%	16%	33%	50%	67%	83%	100%
τ_{max}	323 ± 17	296 ± 5	288 ± 18	379 ± 140	530 ± 15	764 ± 13	239 ± 25
D	7.3 ± 0.7	7.9 ± 0.8	10.8 ± 0.6	22.8 ± 15.1	22.5 ± 3.8	41.7 ± 5.5	17.6 ± 4.7

τ_{max} is the extrapolated maximum turbidity.
 τ_{max} and D are strongly and positively correlated (Pearson $r = 0.90$).
Variable d is length of incubation in days.

310-mL wastewater-based *C. vulgaris* photobioreactors fits quantitatively into the following (Michaelis-Menten kinetics) equation:

$$\tau = \frac{(\tau_{max} * d)}{(D + d)}$$

(1)

where τ_{max} , the maximum turbidity, is obtained by extrapolating d in Eq. 1 beyond 250 days in this study, and D is the number of incubation days needed for τ to reach $\frac{1}{2} \tau_{max}$. All τ_{max} and D values from this study are listed in Table 2.

To devise some means of predicting pH changes during *C. vulgaris* cultivation, given any %WW, a regression analysis (not shown) was performed for each of the plots presented in Fig. 3a-b. The results of the regression analysis suggest that pH variations can be model using the logistic expression below:

$$pH = \frac{pH_{max}}{[1 + Be^{-(C*d)}]}$$

(2)

where pH_{max} is the maximum pH to be expected when d is infinitely large. The distribution of pH_{max} values (Table 3) is found to mirror that of turbidity (Fig. 2). This apparent correlation seems to suggest that pH_{max} is a measure of the carbon requirement for *C. vulgaris* growth and, therefore, an indirect measure of capacity to absorb CO₂ gas. The meanings of B and C are unclear to us at this time. All values of pH_{max} , B , and C obtained here are listed in Table 3. Scatter plots based on this model are presented in Fig. 3c for comparison with experimental plots in Figs. 3a-b.

To determine the range of %WW values and incubation days within which photobioreactor setup such ours can perform above background, PCA was applied to a 7×10 correlation matrix derived from turbidity, or pH values in combination with corresponding %WW and number of incubation days. In Fig. 4a is presented a PCA biplot for the correlation matrix of turbidity changes with days of incubation in various %WW. The %WW markers

Table 3. Averages of Erlenmeyer flasks (Fig. 3a) and beakers (Fig. 3b) values for logistic constants pH_{max} , B and C in modeled growth equation $pH = pH_{max} / [1 + Be^{-(C * d)}]$. These values are obtained by regression analysis of pH as a function of incubation days at each %WW and are reported as mean±standard deviation.

	0%	16%	33%	50%	67%	83%	100%
pH_{max}	8.82 ± 0.05	8.75 ± 0.00	8.75 ± 0.02	8.68 ± 0.02	8.67 ± 0.09	8.61 ± 0.19	8.57 ± 0.08
B	0.09 ± 0.01	0.15 ± 0.00	0.19 ± 0.01	0.23 ± 0.00	0.24 ± 0.01	0.24 ± 0.04	0.26 ± 0.01
C	0.15 ± 0.06	0.22 ± 0.03	0.20 ± 0.04	0.19 ± 0.00	0.15 ± 0.02	0.12 ± 0.04	0.09 ± 0.00

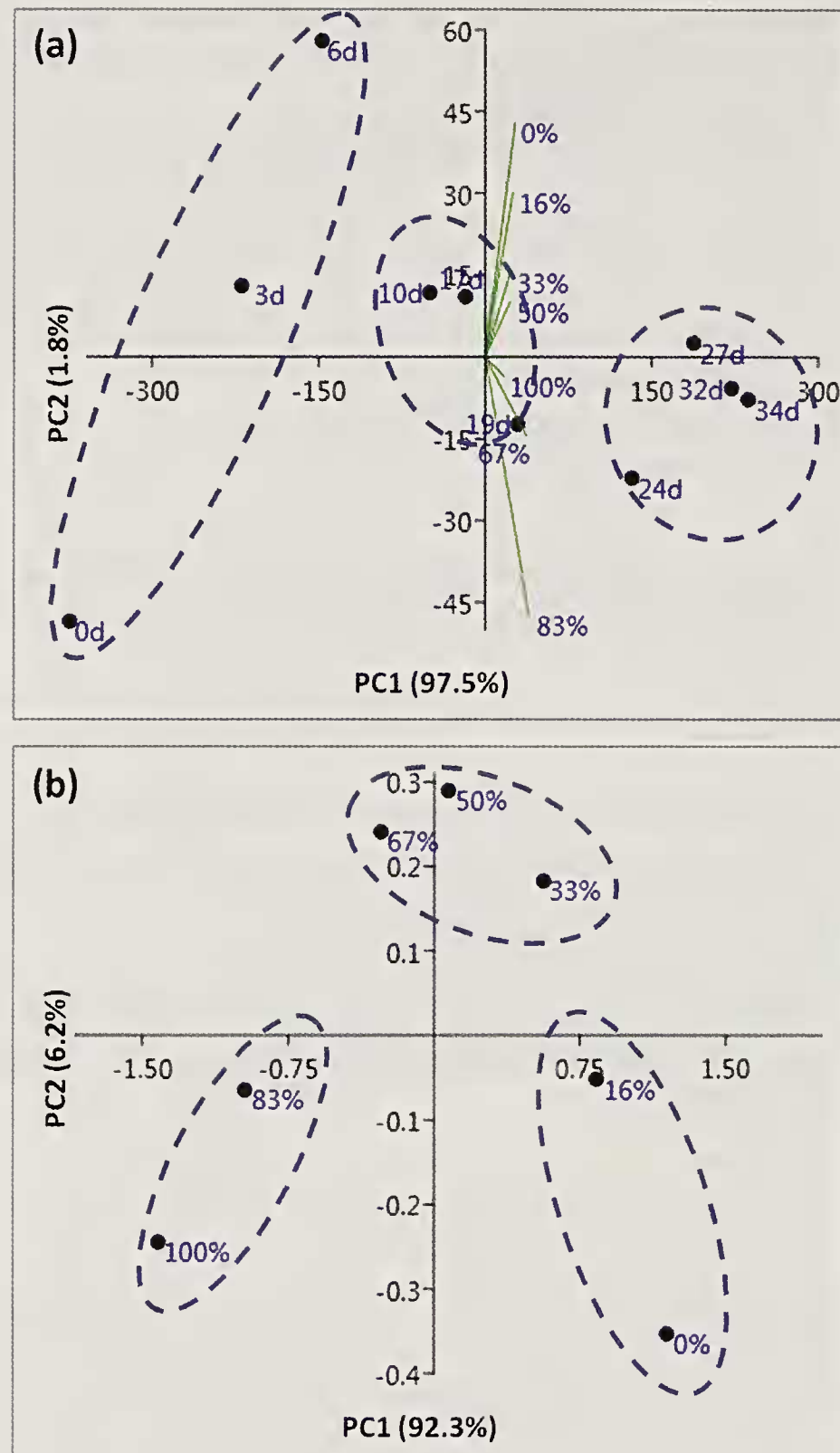


Fig. 4. Scatter plots of two key principal components (PC1 and PC2) from PCA to determine the best combination of %WW, cultivation time (in days, d), and pH values for optimal growth. (a) The loading of incubation days in an ordination space illustrating the influence of %WW as vectors (green lines) on incubation days. (b) The scores of %WW in the same space.

are shown as vectors (green lines), and the incubation days as numbers in 'd' unit. There are two observations which can be made from Fig. 4a. First, that PC1 (which explains 97.5% of variance) strongly correlates with seven of ten incubation days: 0, 3, 6, 24, 27, 32, and 34 days. Of the seven days, PC1 increases with a set of four incubation days longer than 20 (24, 27, 32, and 34 days), and decreases with a set of three incubation days less than 10 (0, 3 and 6 days). The former suggests that turbidity and the set of days 24, 27, 32, and 34 (and days in-between) vary together. The latter implies that incubating *C. vulgaris* in wastewater for 0, 3 or 6 days (and days most likely in-between) will lead to a decrease in turbidity as shown here earlier. This axis (PC1, Fig. 4a) can, therefore, be viewed in similar PCA as a measure of the effect of incubation on algal growth; the larger the value along PC1, the larger the corresponding turbidity value will be. Days between 10 and 20, the 'Goldilocks' days, are not correlated with PC1. Notice also that all %WW vectors (green lines) are accounted

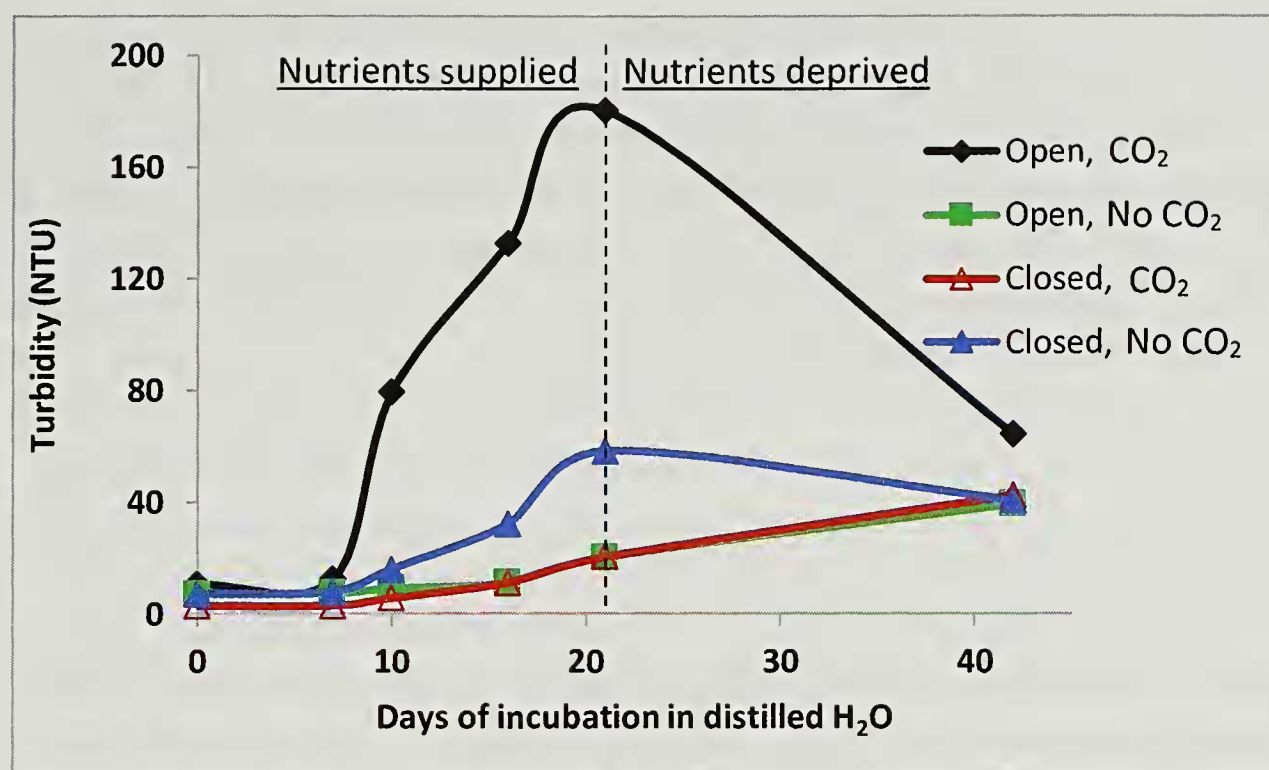


Fig. 5. Determining the level of gaseous CO₂ required for optimal *Chlorella vulgaris* growth rate in distilled H₂O (30 L, Fig. 1a). For the first 21 days (left of vertical dashed line), *Chlorella vulgaris* was supplied with nutrients as described in the text, but deprived of nutrients thereafter. Temperature was maintained at $23.1 \pm 0.2^\circ\text{C}$ over the 42-days of experiment.

for along PC1 with positive loadings. This simply restates the benefit derives from having wastewater in *C. vulgaris* cultures since the larger the values are along PC1, the better.

PC1, however, fails to offer the %WW range that would support optimal *C. vulgaris* growth. This information is provided in the loadings of WW vectors along the second principal component, PC2. It can be seen that PC2, which explains 1.8% of variation, correlates positively with the 0, 16, 33, and 50% WW vectors, but negatively, with the 67, 83, and 100% WW vectors. The 0, 16, 33, and 50% WW cultures were shown earlier in this section to lead to less than optimal turbidity values and hence do not support high *C. vulgaris* yield. The 83% WW culture on the other hand was shown to be the optimal WW level suitable for growth (followed by the 67% WW culture; Fig. 2). Based on these observations, values along PC2 can therefore be considered a measure of the capacity of wastewater to support *C. vulgaris* growth; the small the value, the large the turbidity will be. The 83% WW then stands out as the best growth medium for *C. vulgaris* since it has the lowest value along PC2.

Fig. 4b is a PCA scatter plot for pH. It can be seen that PC1 (which explains 92.3% of variance) is strongly and *positively* correlated with lower %WW (0 and 16% WW), but *negatively* correlated with higher %WW values (83 and 100%). Because evidence shows that lower %WW does not support growth optimally, but 83% is, this principal component can be used as a measure of the best condition for absorbing CO₂ (since the pH of an open photobioreactor is closely linked to the availability of CO₂ in the atmosphere). The smaller the values along PC1, the higher the capacity of the microalgae to absorb CO₂ gas. Note that the clustering of wastewater fractions in Fig. 4b mirrors the distribution of *B* values with %WW in Table 3 which may be suggesting the meaning of constant *B* in Eq. 2.

To understand the role of an open or a closed system against as far as CO₂ supply to a *C. vulgaris* culture is concerned, four separate 30-L tap water-based photobioreactors were set up. Fig. 5 is a summary of the results of these experiments. It can be seen that growth was generally exponential in each photobioreactor over the 21 days of experiment (left side of dashed line in Fig. 5). During this time, the open system registered the best performance

with additional CO₂. The worse performance was observed in the two photobioreactors configured to be open with no additional CO₂ supply or closed with additional supply of CO₂. To reaffirm the importance of non-maternal nutrients to *C. vulgaris*, the four photobioreactors above were incubated for 19 more days without added nutrients. The results, summarized on the right side of Fig. 5 (days between 21 and 40) show that biomass production declined significantly during the period, even in the best photobioreactor configuration (i.e., open with additional CO₂ supply).

Discussion

We have shown that the temporal growth profile of *C. vulgaris* in a wastewater-based photobioreactor follows a nonlinear kinetics (Figs. 2a-b). This is consistent with what others have observed (Ammar 2016). However, that *C. vulgaris* exhibits clearly segmented growth response to wastewater as shown in Figs. 2c-d is a novelty. In this newly discovered profile, the accumulation of *C. vulgaris* biomass declines progressively in low levels of wastewater (0-50% WW), recovers rapid in the 50-83% WW range and then falls precipitously in the 83% to 100% WW range. To explain this profile, it is useful to think of the decline in low levels of wastewater as a reflection of *C. vulgaris* adapting to wastewater-induced shock (Wang et al. 2009). This is justifiable because the burden of adjustment logically increases with the proportion of wastewater in cultures. The rapid biomass increase in the 50-83% WW range then can be thought of as the benefits microalgae derive from wastewater, however miniscule they may be in low %WW cultures is enough to overcome the wastewater-related shock in this %WW range. Beyond this %WW range, the precipitous drop in biomass production may be explained hypothetically as a breach of microalgae cellular biochemistry by high levels of wastewater components. More studies are required to test these hypotheses.

The role that ‘maternal’ nutrients play in growth, which is clearly exhibited in Fig. 2 (0% WW cultures), is worth mentioning here. The fact that *C. vulgaris* grows at all and more so in 100% DH₂O means that maternally stored nutrients (in the cytoplasm and in the inter-thylakoid regions of *C. vulgaris* chloroplast; Safi et al. 2014) are used, we believe, for autonomous growth. This continues until the maternal nutrients are exhausted (in about the sixth day of incubation; Fig. 2d) whereupon wastewater becomes the source of nutrients.

This study finds that the optimal conditions for culturing *C. vulgaris* in an open wastewater-based photobioreactor is $80 \pm 3\%$ WW over a 29-34 incubation days window. It also finds that additional metabolic carbon source beyond atmospheric CO₂ is required to maximize the biomass production in a photobioreactor (i.e., Open, CO₂ system, Fig. 5). The importance of an open photobioreactor system also needs to be emphasized here for two reasons. First, studies show that, without additional source of carbon (industrial CO₂ for example), the capacity of *C. vulgaris* cells to photosynthesize falls which in turn causes a drop in biomass production (Lundquist et al. 2009; Gebreslassie 2013; Raven 2017). Indeed, most photobioreactors fail because of inadequate supplies of metabolic carbon and yet one cheap source of CO₂, the flue gas from power plants, is always available (Gebreslassie, 2013; Kumar et al. 2011). Secondly, O₂ gas, the byproduct of photosynthesis, is toxic to algae (Molina-Grima et al. 2001). It has been shown that microalgae can only tolerate up to 400% of O₂ above the level in O₂ saturated air (Chisti 2007). Having an open wastewater photobioreactor would therefore reduce toxicity by allowing O₂ produced during

photosynthesis to escape (while still leaving enough for microbes to use in the breaking down of organic matter in wastewater; Azov et al. 1982; Abdel-Raouf et al. 2012).

This study identified five elements of a wastewater-based *C. vulgaris* photobioreactor (τ_{max} , pH_{max} , D , B , C) which could be useful for forecasting productivity even though the meanings of B and C is still to be determined. For any chosen %WW, we have introduced (i) the concept of the minimum days (D) a static photobioreactor would need to produce biomass above background, (ii) the concept of pH_{max} , the upper ceiling of pH which can be used to predict the capacity of a wastewater-based *C. vulgaris* photobioreactor to absorb CO₂ in an open photobioreactor system, and (iii) the concept of τ_{max} , the upper ceiling of biomass production when incubation is continued indefinitely beyond the minimum days, D (i.e., $d \gg D$; Eq. 1). One property of τ_{max} needs elaboration. Unlike pH_{max} and D , the values of τ_{max} cannot be obtained directly by *C. vulgaris* cultivation because of the limits imposed on production by the carrying capacity of photobioreactors and the long incubation time required to achieve it. For example, the average carrying capacity of our 310-mL system was 340 NTU (the mean of 333 NTU and 347 NTU; Fig. 2a and Fig. 2b, respectively) and yet τ_{max} was determined to be 773 NTU in a 83% WW culture (after 550 years of incubation).

A number of factors were confirmed in this study to have significant consequence on the effectiveness of using microalgae to polish municipal wastewater. For example, we find that efficient removal of nutrients from wastewater as measured by increase in turbidity depends on constant agitation of the *C. vulgaris* cultures. This is of consequence in municipalities where microalgae have been adopted to polish wastewater, but agitation has not been incorporated into their photobioreactor designs (Craggs et al. 2003). Exacerbating this problem is the fact that the C:N ratio in a typical municipal wastewater is low when compared to the carbon requirements of microalgal biomass. This low C:N ratio limits the capacity of microalgae to produce biomass in wastewater (Benemann 2003). This imbalance is usually manifested as elevated pH levels during incubation (resulting from the use of bicarbonate ions as a CO₂ source for algal photosynthesis, releasing hydroxide ions; Craggs et al. 2011) which in turn prevents growth of both microalgae and bacteria which degrade the organic compounds in wastewater (Azov et al. 1982). This scenario was observed in photobioreactors which were not supplied with additional CO₂ (Figs. 3, 5). Boosting carbon supply to a wastewater-based culture would not only enhance microalgal yield as has been shown here, it would also fulfill a goal to scrub CO₂ gas from sources beyond the atmosphere. Note that the main function of microalgae for polishing municipal wastewater is to remove N, and not P (Choi and Lee 2015). This is because the large N:P ratio requirement for biomass production in microalgae means no additional microalgal biomass production is needed above that required to assimilate N to remove P from municipal wastewater (Lau et al. 1995; Benemann 2003; Craggs et al. 2011).

The environmental remediation approach proposed in this study will results in massive amounts of algal biomass, the fate of which must be addressed appropriately. Fortunately, some means for doing so already exist even though they were not designed with addressing environmental issues in mind. For example, microalgae biomass has been turned into chemical feedstock for biodegradable plastics (Lambert and Wagner 2017), lubricants (Ruiz et al 2016), fertilizers (Uysal et al. 2015), and pharmaceutical/nutraceutical products (Jha et al. 2017). In the skin-care natural product industry, the capacity of microalgae to developed sun blocking agents to protect and heal themselves from the damaging impacts of exposure to solar radiation and other environmental hazards has been harnessed to protect human skin (Stolz and Obermayer 2005). In some parts of the world, microalgae have

been used sustainably as sources of animal feed because of their ability to concentrate carbohydrates, proteins and vegetable oils, micronutrients, vitamins, and valuable pigments (Lambert and Wagner 2017). In an application incompatible with our goal, microalgae biomass has also been used as human dietary supplements (Yaakob et al. 2014). And of recent, a technology has emerged that has potential to convert microalgal biomass into a host of products. Currently, this proprietary technology employs high energy electron beams to convert cellulose in corn cobs to edible sugar, alcohol, biodegradable plastics and several other products on industrial scale³. Microalgal biomass could be treated similarly.

Capturing and storing CO₂ emission is one feasibly way of mitigating climate change. How the captured CO₂ is stored however will have an impact on the effectiveness of long-term sequestration. For example, the ocean's capacity to absorb and store huge quantities of CO₂ could be used to sequester CO₂. This though comes with environmental cost and legal problems. First, several studies have shown that sequestering CO₂ in the ocean leads to global warming which ultimately leads to excessive plant growth. As pointed out in the Introduction section, this excessive plant growth creates dead zones in the ocean (Shaffer 2010). Secondly, the Clean Water Act makes it impossible to sequester CO₂ into the ocean.^{4,5} The Act prohibits point source discharges into navigable waters in the USA without a permit; it also ensures that marine environments will not suffer unreasonable degradation or irreparable harm from anthropogenic activities. One alternative to the ocean is geological sequestration (Shaffer 2010). This alternative involves injecting captured CO₂ into geologic environments (Aarnes et al., 2010; Plasynski et al., 2011). However, the alternative also comes with one major environmental concern, that of leaks from storage sites.

Conclusions

We have demonstrated that a microalgae-based photobioreactor can offer a potential solution to lowering the level of atmospheric CO₂ while cleaning nutrients-laden municipal wastewater at the same time. By culturing *C. vulgaris* in various photobioreactor systems containing wastewater, we have shown that wastewater plays two competing roles during *C. vulgaris* growth: a mostly detrimental and dominant role at low levels where we believe wastewater interferes with the metabolic mobilization of the maternal nutrients required for the initial growth, and a mostly supportive role that is very evident in the 50-83% WW range cultures. As such, the optimal conditions for *C. vulgaris* to absorb atmospheric CO₂ are found to be 78-83% dilution of wastewater (the baseline is 33% WW) and 24 days of incubation in open air. We have also shown that principal components analysis can be applied to rudimentarily acquired data such as ours to aid in determining the optimal wastewater requirement of a photobioreactor. The size of the chosen photobioreactor would be limited by the carrying capacity of photobioreactor.

³ Stahl, L. 2019. The unlikely, eccentric inventor turning inedible plant life into fuel. CBS 60 Minutes. Available from: <https://www.cbsnews.com/news/marshall-medoff-the-unlikely-eccentric-inventor-turning-inedible-plant-life-into-fuel-60-minutes/>. Accessed 15 January, 2019.

⁴ Cleanwat.001. 2002. Federal Water Pollution Control Act. Available from <https://www.epa.gov/sites/production/files/2017-08/documents/federal-water-pollution-control-act-508full.pdf>. Accessed 15 January, 2019.

⁵ Heinrich J. 2002. Legal implications of CO₂ ocean storage. Available from: https://sequestration.mit.edu/pdf/Legal_Implications_Ocean_Storage.pdf. Accessed 15 January, 2019.

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